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13. ABSTRACT ***(Maximum 200 Words)***
Mammary gland development in the galanin knockout mouse has been the focus of activity during the previous year. The galanin knockout was found to show failure of mammary development during the first pregnancy which resulted in a 100% failure of lactation. Subsequent pregnancies resulted in normal development and successful lactation. Mammary gland recombination/transplantation showed that this effect was not mammary epithelial cell autonomous, and did not require stromal galanin, ruling out an autocrine role for galanin. Analysis of the galanin receptor 1 knockout revealed no lactational deficit, ruling out an autocrine role for galanin via this receptor. An endocrine role via the other receptors is currently under investigation using whole mammary gland organ culture Similarity to the prolactin knockout phenotype suggested prolactin as a mediator of an indirect effect of galanin, and attempts to rescue developmental failure with osmotic minipump administration of prolactin were successful. The expression pattern of galanin and its receptors is under investigation at various stages of mouse mammary development. Studies in human material have used cell lines to date, where the expression patterns of galanin and its receptors have been determined, allowing the selection of suitable cell lines for in vitro analysis of the potential growth promoting action of galanin. Experiments using 96 well plate MTT proliferation assays have proved difficult due to variability of response, and ongoing work has shifted to the use of clonogenic assays. The formation of arrayed human breast cancer biopsies has begun to allow the analysis of galanin expression during disease progression. The mouse work is currently being prepared for publication. During these experiments observations were made regarding the role of the mouse stroma in the control of mammary ductal branching patterns and a brief communication has been prepared and submitted for publication.

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INTRODUCTION

Galanin, a neuropeptide previously thought to be restricted to the central and peripheral nervous system is implicated in the growth control of a number of cell types. Recently we showed that galanin is expressed by human breast cancer cell lines under the control of estrogen and progesterone. In cell lines derived from small cell lung cancer, a tumour which relapses rapidly with an aggressive phenotype, galanin caused rapid mobilisation of calcium, accumulation of inositol phosphate and activation of the MAP kinase isoform p42 via a protein kinase C dependent mechanism, producing increased clonal cell growth in soft agar. By analogy galanin may also be a growth factor for both the normal breast and breast cancer. This proposal has the objective of testing this hypothesis. The specific aims are to examine the role of galanin in normal mammary gland development, human breast cancer and experimental carcinogenesis.

BODY

The research proposal submitted contains the following objectives (in bold print). Our progress to date with each of these objectives is indicated in plain type.

Specific Aim A: Examination of the role of galanin in normal mammary gland development.

1. **The normal expression patterns of galanin and the galanin receptor subtypes will be examined using *in situ* hybridisation of normal mouse mammary glands at the major developmental stages of puberty, pregnancy, lactation and involution.**

   Table 1 outlines the progress made to date. Tissue collection is almost complete and whole mount histology of contraleteral glands has been undertaken for most samples. Vectors for galanin and receptors have been obtained and test in situ hybridisations have successfully been performed. A tissue micro array (see A2 below) approach will be taken to simplify this task.

2. **The expression pattern of galanin and its receptors by the normal human breast will be examined by *in situ* hybridisation of specimens selected from a panel of 103 breast biopsies obtained at reduction mammaplasty (60**

biopsies) or from non involved breast obtained at radical mastectomy (43 biopsies).

The advent of tissue micro arrays since the commencement of this project has greatly simplified this task. A tissue micro array fabrication device has recently been purchased by the translational research laboratory within the Cancer Research Program and fabrication of arrays will commence in the latter half of this year, and will be made available to us. This will allow this task to proceed more efficiently.

3. **Galanin knockout mice, obtained by collaboration with David Wynick and now at the Garvan Institute, will be used to examine mammary development at puberty, pregnancy, lactation and involution, by whole mount and histological techniques, to determine if galanin is involved in mammary development.**

These experiments have been completed. Table 1 outlines the stages examined to date. Defects in mammary development during puberty and the first pregnancy have been identified in galanin knockout (ko) females (Figure 2). During puberty ductal side branching fails in the galanin ko. During the first pregnancy lobuloalveolar development fails, and the first litter dies from dehydration/malnutrition due to lactational failure. Lactation is successful following the second pregnancy.

4. **To determine if the effects defined in A3 are due to the loss of galanin in the mammary gland, or to indirect effects due to the loss of galanin from other endocrine organs, transplant of mammary glands from wild type and knockout animals will be made to endocrinologically normal hosts and examined by whole mount and histochemistry at puberty, pregnancy and involution.**

These experiments have been completed. Transplant of galanin ko epithelium to cleared wild type (wt) fat pads allows mammary development to proceed normally at the first pregnancy (Figure 2). In these experiments ductal side branching was

identical between ko and wt epithelium, but both exhibited very little side branching, preventing conclusions regarding the pubertal defect. To determine if galanin in the stroma played any role mammary recombinations were made, allowing wt epithelium to be directed by wt or ko mammary stroma (Figure 3). Again no defect in development was seen in recombinations using ko stroma. These experiments show that galanin plays no autocrine role in mammary development- the cause of the defect in galanin kos must lie elsewhere.

Comparison of the recombination and cleared fat pad experiments showed remarkable differences in the level of ductal side branching achieved in virgin animals. The stroma used in these experiments was from 129 animals in the former case and C57BL/6 animals in the later technique. As these strains exhibit different levels of side branching we hypothesised that it was the mammary stromal compartment that underlay this effect, and subsequent experimentation confirmed this. These experiments are detailed in the attached manuscript currently under review for publication.

The failure to transplant the galanin ko phenotype leaves two possibilities, galanin plays a direct endocrine role, or galanin plays an indirect role. Experiments examining the direct role are detailed below in 5A. The similarity between the galanin ko and the prolactin receptor ko suggest prolactin as a possible indirect mediator of galanin action in the mammary gland. To test this hypothesis we formed a collaboration with Dr. Ameae Walker, UC Riverside, who has supplied us with prolactin and a phosphoprolactin mimic to administer via osmotic minipump. Preliminary results to date indicate that prolactin administration can rescue the first lactation failure in galanin kos.

5. **The possibility of mammary galanin acting as an endocrine factor to influence mammary development will be examined using conditional galanin knockout animals obtained by collaboration with David Wynick. These animals will have only the mammary gland galanin gene ablated. Hormonal profiles will be measured. A single normal mammary gland will be transplanted to these animals and hormonal profiles again measured. The development of the transplants will be assessed at puberty, pregnancy**

and involution and compared to a normal mammary glands transplanted to normal hosts.

The conditional galanin ko animals are not yet available from Dr Wynick. This is the best model to examine the potential endocrine role of mammary galanin. In the interim we have formed a collaboration with Dr. Barbarra Vonderhaar, NCI Bethesda to examine the effects of galanin administration in vitro mammary gland development, an organ culture technique which has been developed in Dr Vonderhaars laboratory. We have no results to report to date.

Specific Aim B: Examination of the role of galanin in human breast cancer.

1. **The panel of breast cancer cell lines used to examine galanin gene expression will be screened for galanin receptor subtype expression by PCR. Cell lines selected on the basis of receptor expression will be treated with galanin and cell cycle phase distribution, cell growth and colony formation will be measured.**

Examination of galanin and galanin receptor gene expression in the panel has been completed and the results are indicated in Table 2. Using this information a number of cell lines have been selected, and the potential growth promoting effects of galanin, and growth retarding effects of galanin neutralising antibodies have been assessed in a 96 well plate MTT assay. Five repeats of this assay have failed to produce consistent results, due we believe to the difficulty in removing serum by repeated washing from the small wells without loosing the cells. Rather than persist we have elected to repeat the galanin addition experiments using a colony formation assay. These experiments will be initiated later this year, and if successful the 96 well approach will be pursued to allow the use of neutralising antibodies.

2. **Breast tumours selected on the basis of phenotype from a large panel of specimens will be used to determine which cell types express galanin and its receptors by in situ hybridisation. Results will be related to the normal expression patterns defined in A1 and A2, and to tumour phenotype,**

steroid hormone receptor expression, markers of poor prognosis, proliferative and apoptotic markers.

This will be pursued along with A2 by tissue microarray. No progress to date

3. **The expression of galanin and its receptors by breast cancers will be measured by RT-PCR and correlated with disease outcome in a large panel of breast cancers for which RNA and 74 month average post diagnosis clinical follow-up are available.**

This will be pursued along with A2 by tissue microarray. No progress to date.

Specific Aim C: Examination of the role of galanin in experimental carcinogenesis.

1. **Conditional galanin knockout mice, lacking galanin expression in the mammary glands, will be treated with DMBA, a chemical carcinogen requiring additional hormonal stimulus for full activity. Tumour latency, frequency, histological grade and metastasis will be compared between genotypes.**

No progress to date. the conditional knockout animals are not yet available

2. **A transgenic mouse expressing galanin under the control of the mouse mammary tumor virus promoter will be constructed and examined for altered rates of tumorigenesis, either spontaneously in virgin and multiparous animals, or in conjunction with DMBA treatment. Tumour latency, frequency, histological grade and metastasis will be compared between genotypes.**

A finding that the phenotype in the galanin ko is indirect via modulation of prolactin secretion would made the results of this study difficult to interpret, and so construction of a galanin transgenic mouse has been delayed until the mechanism of galanin action is better understood. The resources that would be

consumed by this subproject may be better reallocated to the translational and cell culture experiments if galanin is shown to act indirectly on morphology.

Discussion

The results to date in the galanin ko mouse suggest that galanin plays an indirect role in mammary gland development. A manuscript detailing these experiments is in preparation. These findings leave open the question of why the mammary gland expresses galanin. It may be an endocrine factor linking the mammary epithelium and pituitary prolactin production- but this hypothesis requires the production of a mammary specific galanin knockout which is not yet available.

The role of galanin in human cancer remains to be investigated, and will be the subject of the next two years work. Recent technological developments in this area will speed this work, allowing a more powerful analysis than outlined in the original application.

Overall we are pleased with progress to date, after 12 months we have almost completed our investigations of the galanin knockout mouse and have a solid data set to publish. We can now focus on the analysis of galanin in human cancer.

Key Research Accomplishments

- Complete analysis of the role of galanin in normal mammary gland development.
- Initiation of translational studies regarding the role of galanin in human breast cancer.

Reportable Outcomes

1. Abstracts of presentations at the following meetings
    a. Australian Society for Medical Research, Leura NSW Nov. 1999
    b. Gordon Conference on Prolactin. Ventura Ca Feb 2000
    c. Keystone Breast and Prostate Meeting, Lake Tahoe NV Mar 2000

2. Manuscript
    Mouse strain specific patterns of mammary ductal branching are elicited by the stroma. Naylor and Ormandy. Submitted.

Conclusions

1. Galanin modulates mammary gland development via control of pituitary prolactin release.

So what?

The Nurses Healthy Study (Hankinson 1999) has shown that serum prolactin levels in the top quartile are associated with a 2-3 fold increase in the relative risk of breast cancer, similar to the risk associated with increased serum estrogen levels (Hankinson 1998). The effect of prolactin is independent of estrogen. Thus factors influencing serum prolactin levels can influence susceptibility to breast cancer. Galanin is such a factor.

The influence of galanin in human breast cancer will be examined in coming years.

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Hankinson SE. Willett WC. Manson JE. Colditz GA. Hunter DJ. Spiegelman D. Barbieri RL. Speizer FE. Plasma sex steroid hormone levels and risk of breast cancer in postmenopausal women [see comments]. [Journal Article] Journal of the National Cancer Institute. 90(17):1292-9, 1998 Sep 2.

Appendices

Abstracts

a. Australian Society for Medical Research, Leura NSW Nov. 1999

b. Gordon Conference on Prolactin. Ventura Ca Feb 2000

c. Keystone Breast and Prostate Meeting, Lake Tahoe NV Mar 2000

Manuscript

Mouse strain specific patterns of mammary ductal branching are elicited by the stroma. Naylor and Ormandy. Submitted.

Galanin: A possible role in development and carcinogenesis of the mammmary gland

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Galanin is a neuropeptide that is reported to have a role in the control of a number of physiological functions including neuroendocrine responses and mitogenesis. We are investigating the possible role of galanin in normal and cancerous breast. In normal breast galanin may act directly as a growth factor. Galanin and prolactin receptor knockout mice display similar mammary gland phenotypes of reduced ductal branching and alveolar density. These actions of galanin on normal breast may be mediated indirectly, as galanin induces prolactin secretion from the pituitary.

In human breast cancer there is evidence to suggest that galanin acts directly as a growth factor. RT-PCR for expression of galanin and its receptors (GALR1,2,3) was performed on a panel of 18 human breast cancer cell lines and 4 normal epithelial breast cell strains. Galanin mRNA was identified in 17/22 cell lines, while GALR1 was identified in 5/22 and GALR2 was identified in 8/18 cancer cell lines and 3/4 normal cell strains. Interestingly, galanin receptor expression was strongly associated with ER negative cell lines where 7/10 ER negative cell lines expressed at least one receptor, while 3/8 ER positive cell lines expressed galanin receptor mRNA.

Ongoing transplantation studies aim to determine whether galanin acts directly or indirectly on breast epithelium to influence mammary gland development.

Galanin: A possible role in the development of the mammary gland.

Matthew Naylor[1], Tiina Iismaa[2], David Wynick[3] and Christopher Ormandy[1]. [1]Development Group-Cancer Research Program, and [2]Neurobiology Program, The Garvan Institute of Medical Research, St Vincent's Hospital, Sydney, NSW, 2010, and [3]Department of Medicine, Bristol University, Marlborough St, Bristol BS2 8HW United Kingdom.

Galanin is a peptide involved in the control of a number of physiological functions including neuroendocrine responses and mitogenesis. We have observed that galanin is expressed by human breast cancer cells (Cancer Res 58:1353), suggesting a possible role for galanin in normal mammary development and carcinogenesis. Direct autocrine or paracrine actions of galanin are possible as mammary epithelial cells coexpress both ligand and receptor. RT-PCR for galanin and its receptors (GALR1,2,3) was performed on a panel of 18 breast cancer and 4 normal breast epithelial cell lines. Galanin mRNA was identified in 17/22 cell lines, while GALR1 was identified in 5/22 lines, GALR2 in 11/22 lines and GALR3 in 6/22 lines. Normal mouse mammary gland also coexpressed galanin and its receptors. An indirect role for galanin is indicated via control of pituitary prolactin secretion (PNAS 95:12671), and galanin and prolactin receptor knockout mice (Gene Dev 11:167, Dev Biol 210:96) display similar mammary gland phenotypes of reduced mammary ductal branching and lactational failure following the first pregnancy. To determine if galanin has an autocrine/ paracrine role in mammary development, transplantation to $Rag1^{-/-}$ hosts of recombined $GAL^{-/-\ \&\ +/+}$ mammary epithelium and stroma was performed. On the basis of morphology, these experiments showed no autocrine/ paracrine role for galanin in normal mammary gland development. We are currently investigating whether galanin acts in an endocrine manner to influence mammary gland development.

Mouse strain-specific patterns of mammary ductal branching are elicited by the stroma.

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Running Title: Stromal control of ductal side-branching

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Key Words: stroma; mesenchyme; epithelium, endocrinology, development; tissue recombination; mammary gland; transplantation

ABSTRACT

Variations in mammary ductal side-branching patterns are known to occur between different strains of mice and the rate of spontaneous cancers are increased in those strains which show highly side-branched mammary architecture. The cause of the variation in ductal side-branching between mice strains is unknown, but epithelial, stromal and endocrine factors have been implicated. To define the mammary elements responsible for controlling ductal side-branching patterns, we formed recombined mammary glands from epithelial and stroma elements taken from highly side-branched 129Ola/SvPas and poorly side-branched C57BL/6J mammary glands, and transplanted them to $Rag1^{-/-}$ hosts on the C57BL/6J background. When 129Ola/SvPas epithelium was recombined with C57BL/6J stroma the poorly side-branched C57BL/6J ductal pattern was observed. 129Ola/SvPas epithelium recombined with 129Ola/SvPas stroma reproduced the highly side-branched 129Ola/SvPas ductal pattern, while C57BL/6J epithelium recombined with 129Ola/SvPas stroma, also resulted in development of the highly side-branched ductal pattern. All transplants utilized the same C57BL/6J endocrine background. These studies demonstrate that strain differences in the mammary stroma are responsible for the strain-specific ductal side-branching patterns and that the epithelial strain or endocrine background play no part. Performing mammary epithelium or recombination transplants without regard to the stromal strain, or use of stroma of mixed genetic background, may lead to the misinterpretation of results.

INTRODUCTION

Post-natal changes in the architecture of the mammary gland occur during puberty, pregnancy, lactation and weaning. Recently the construction of various gene knockout animals and subsequent transplantation studies have facilitated dissection of the signalling components required for these events, and a host of factors including hormones, growth factors, transcription factors and cell cycle regulatory proteins have been demonstrated to be required for these developmental changes (Hennighausen and Robinson, 1998). Transplantation and mammary recombination techniques have been used to define the mammary elements which these factors influence. Factors may act via the stroma eg. activin/ inhibin, ERα, EGFR and family ligands (Robinson and Hennighausen, 1997; Cunha *et al.*, 1997; Wiesen *et al.*, 1999), while others act via the epithelium eg. PR and PRLR and their ligands (Brisken, 1998; Brisken, 1999). Others may have actions via indirect mechanisms (Brisken, 1999).

Variations in ductal side-branching occur between different mouse strains. The C3H/HeNHsd, Balb/c and 129 substrains (Ola, Pas and SvJ) (129) display a highly side-branched mammary architecture, while Nude/ onu (nude) and C57BL/6J (C57) strains have very little ductal side branching (Gardner and Strong, 1935). The factors controlling this strain variation in mammary side-branching are unknown. Strain-specific branching patterns are also related to the susceptibility of different strains to mammary tumors; strains which display a highly side-branched mammary architecture have an increased rate of spontaneous cancers compared to strains with less ductal side-branching and alveolar formation (Gardner and Strong, 1935; Husby and Bittner, 1946). These observations suggest that the mechanisms controlling the differences in mammary side-branching between strains may also be involved in the development of cancer.

Mammary recombination transplant techniques were employed to determine whether strain variation in mammary side-branching is due to genetic differences in the stromal, epithelial or endocrine compartments. We demonstrate here that differences in mammary side-branching observed between strains are determined by the stromal element regardless of strain variation in the epithelial component or endocrine environment. Thus in mammary transplantation and recombination experiments, choice of the stromal strain used becomes critical. The use of stromal strain that limits side branching, or stroma from mice of mixed genetic background, may produce misleading or highly variable results.

RESULTS

Different strains of mice show variations in the level of ductal side-branching. The mice used in this study were the poorly side-branched C57BL/6J strain (Fig. 1A) and the highly side-branched 129 Ola/SvPas strain (Fig. 1B). Epithelium from 129 mice was transplanted into C57 strain $Rag1^{-/-}$ mice using the cleared fat pad technique devised by DeOme and co-workers (DeOme *et al.*, 1959). The pattern of mammary development observed was that of the endogenous C57 gland (Fig. 1C), suggesting that the strain of the stroma, or host endocrinology, but not epithelium, controlled side-branching patterns.

Recombination of stromal elements was performed to determine whether or not the stroma was responsible for controlling strain differences in side-branching. Repetition of the cleared fat pad transplants using the recombination technique reproduced the previous result; 129 epithelium into C57 stroma produced poorly branched C57 architecture (Fig. 2A). However, transplantation of 129 epithelium into 129 stroma resulted in development of the highly branched 129 architecture (Fig. 2B). Similarly, in recombinations of C57 epithelium with 129 stroma, 129 mammary architecture was seen (Fig 2C). In all transplants the endocrine system was C57 and comparisons were made between transplants grown in the same $Rag1^{-/-}$ animal, excluding endocrinology as a controlling factor. These studies, summarised in Table 1, identify mammary stroma as the principle component responsible for controlling strain-specific ductal side-branching patterns. In mammary recombination transplants the area of fat pad for ductal outgrowth is reduced compared to that of the cleared fat pad technique. Transplants of identical combinations of epithelium and stroma were performed using the recombination and cleared fat pad transplant techniques to investigate whether the level of branching observed in the mammary recombination technique is an artefact of epithelial crowding. The absolute density of ductal outgrowth from both the transplant techniques and the endogenous gland were observed to be identical (data not shown), confirming our conclusions regarding the controlling role of the stroma.

DISCUSSION

Strain differences in the levels of steroid hormones, particularly small variations in progesterone during the estrous cycle, were thought to explain the variation in branching patterns observed between mouse strains. This hypothesis is supported by recent work which demonstrates a crucial role for progesterone in side branch formation. Progesterone receptor A transgenic mice show a hyperbranched mammary architecture (Shyamala *et al.*, 1998) while progesterone receptor knockout mice show an absence of mammary side branches (Lydon *et al.*, 1995) due to the loss of epithelial progesterone receptor (Brisken *et al.*, 1998; Brisken *et al.*, 2000). Work presented here, however, demonstrates that strain differences in endocrinology cannot explain the differences in side-branching.

The epithelial transplants (Fig. 1C) and recombination transplants (Fig. 2) demonstrate that the strain-specific patterns of ductal side-branching are independent of the epithelial component, supporting conclusions reached in other mouse strains (Yant *et al.*, 1998). The recombination transplants shown in Figure 2 conclusively demonstrate that it is the stroma which elicits the effect, and that strain differences in progesterone levels, or any other endocrine factor, do not cause the observed differences in ductal side-branching between strains.

These experiments expose a second control mechanism of ductal side-branching that resides within the stroma. The ability of stromal factors to induce gland-specific morphogenesis is well established (Sakaura *et al.*, 1976; Sakaura *et al.*, 1982; Cunha *et al.*, 1995). Sakakura and colleagues demonstrated mesenchyme-dependant epithelial morphogenesis and epithelium specific cytodifferentiation using salivary and mammary heterotypic recombinations. It is tempting to speculate that the same mesenchyme factors are responsible for both the ability of salivary mesenchyme to induce salivary gland epithelial morphology in mammary epithelium, and for the strain-specific differences in epithelial branching patterns. The components within the stroma that control strain-specific ductal side-branching are unknown. Recent work has demonstrated the crucial role of the Wnt proteins in ductal side-branching (Buhler *et al.*, 1993; Bradbury *et al.*, 1995; Brisken *et al.*, 2000) and a plausible hypothesis is that strain-specific stromal levels of expression of the Wnt family of proteins may play a

role in the observed variations in mammary ductal branching patterns among strains, and importantly may influence strain variation in the incidence of mammary tumors.

Transplantation and mammary recombination techniques have become an invaluable tool in the elucidation of the role of various components controlling mammary gland development. A variety of different transplantation techniques have been described depending on the nature of the transgenic animal and the questions being addressed (DeOme *et al.*, 1959; Cunha *et al.*, 1997; Brisken *et al.*, 1998), but the importance of choosing the correct host environment in which to monitor mammogenesis following transplantation has not been well investigated. $Rag1^{-/-}$ mice serve as a good host system for transplantation as they have a normal endocrine environment (Brisken *et al.*, 1999), compared to nude mice (Kopf-Maier and Mboneko, 1990). This study demonstrates the importance of using stroma from a highly side-branched strain when side-branching density is the endpoint under examination. For example, if 129 epithelium was transplanted into C57 stroma, the maximum branching that can be produced is that of the poorly branched C57 stroma, potentially masking transplant-induced changes in side-branching morphology. Furthermore, in our experience, branching patterns vary between individual hosts when the stroma from a mixed 129/ C57 strain is used. Presumably the variation in these outbred individuals is due to the mixed genotype of the stroma.

EXPERIMENTAL PROCEDURES

Mice

129 mice used in these experiments were the 129 Ola/SvPas mixed sub-strain. $Rag1^{-/-}$ mice (Momberts *et al.*, 1992) of the C57BL/6J strain were purchased from Animal Resource Centre, Perth, Australia. All animals were housed with food and water *ad libitum* with a 12 hr day/night cycle at $22^{o}C$ and 80% relative humidity.

Mammary epithelium transplants

Mammary epithelium transplants were performed as described (DeOme *et al.*, 1959). In brief an approximately 1 mm^3 section of mammary gland was excised from 12 week old 129 or C57 strain donors from between the nipple and lymph node of the 4th mammary gland. The opposite 4th mammary gland was taken for carmine stained histology as a reference. The donor mammary epithelium was

transplanted into the cleared mammary fat pad of 3 week old Rag1$^{-/-}$ mice. The transplanted gland and an endogenous gland were examined by histology or whole mount analysis 12 weeks post surgery.

Mammary recombination transplants

Mammary recombination transplants were performed by inserting donor epithelium (129 or C57 strain) prepared as described above, into the excised fat pad of either 129 or C57 3 week old mice cleared of endogenous epithelium. The recombined mammary epithelium-stroma complex was grafted between the abdominal cavity and skin, placing the transplant between the 3rd and 4th mammary glands of 3 week old Rag1$^{-/-}$ mice (Brisken *et al.*, 1998). The following recombinations of mammary epithelium (Ep) and stroma (St) were performed: 129 Ep & C57 St; 129 Ep & 129 St; C57 Ep & 129 St; and C57 Ep & C57 St. Recombined transplants were examined by whole-mount histology at 6 weeks post surgery.

Histology

Mammary whole mounts were performed by spreading the gland on a glass slide and fixing in 10% formalin solution. Glands were defatted in acetone before carmine alum (0.2% carmine, 0.5% aluminium sulfate) staining over night. The whole mount was dehydrated using a graded ethanol series followed by xylene treatment for 60 minutes and storage in methyl salicylate (Bradbury *et al.*, 1995).

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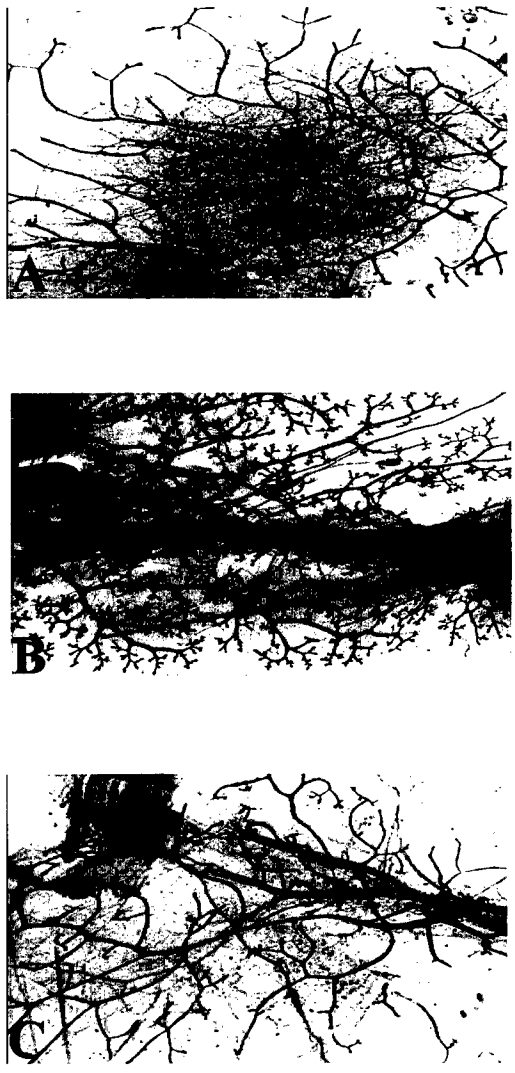
Table 1. Summary of results.

Technique	Epithelium	Stroma	Endocrinology	Side Branching
Endogenous	C57	C57	C57	low
Endogenous	129	129	129	high
Epithelial	129	C57	C57	low
Recombination	129	C57	C57	low
Recombination	129	129	C57	high
Recombination	C57	129	C57	high

FIGURE LEGENDS

Figure 1. Whole mount of 4th mammary gland from 12 week old mice (A) C57BL/6J strain. (B) 129 Ola/SvPas strain. Whole mount of mammary epithelial transplant (C) 129 Ep / C57 St. 8x magnification.

Figure 2. Whole mounts from recombination transplants. (A) 129 Ep / C57 St. (B) 129 Ep / 129 St. (C) C57 Ep / 129 St. (D) Endogenous C57 Ep / C57 St. 8x magnification.



Naylor and Ormandy. Stromal control of ductal side branching Figure 1.